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Original Article

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MiR-181a-5p regulates 3T3-L1 cell adipogenesis by targeting *Smad7* and *Tcf7l2*

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Abstract

MicroRNAs are highly conserved non-coding small RNAs participating in almost all kinds of biological activities. MiR-181a has been reported to be involved in the differentiation of porcine primary preadipocytes, but the profound effect of miR-181a-5p on 3T3-L1 adipocyte differentiation and proliferation is still unclear. In this study, we found that supplementation of miR-181a-5p in 3T3-L1 cells significantly promoted the adipogenesis and inhibited cell proliferation with increased expression of adipogenic marker genes including peroxisome proliferator-activated receptor gamma (*Pparγ*), CCAAT/enhancer-binding protein alpha (*C/ebpα*), fatty acid-binding protein 4 (*Fabp4*), and Adiponectin, accompanied by an accumulation of lipid droplet, an increase of triglyceride content, and a decrease of cell proliferation. Furthermore, by using the luciferase assay, *Smad7* and *Tcf7l2*, two important members of transforming growth factor-β (TGFβ) and Wnt signaling pathway, were proven to be the direct target genes of miR-181a-5p. Moreover, supplementation of miR-181a-5p in 3T3-L1 cells altered the expressions of proteins involved in the TGFβ signaling pathway, such as TGFBR1, p-SMAD3, SMAD4, c-MYC, and p15. Taken together, these results indicate that miR-181a-5p promotes 3T3-L1 preadipocyte differentiation and adipogenesis through regulating TGFβ/Smad and Wnt signaling pathway by directly targeting *Smad7* and *Tcf7l2*.

Key words: miR-181a-5p, adipogenesis, Smad7, Tcf7l2

Introduction

In recent years, the prevalence of overweight and obesity in China has been markedly increasing based on the China Health and Nutrition Surveys [1,2]. Studies have indicated that obesity is a complex metabolic disorder that is often associated with all kinds of diseases, such as type 2 diabetes mellitus, endometrial disorder, hypertension, coronary heart disease, and hepatic steatosis, and is also an important risk factor for cancers [3–6]. It has been demonstrated that in a state of obesity accompanied by the gradual expansion of adipose tissue, the whole body energy balance can be destroyed, which further increases the risk of insulin resistance, hypertension, and dyslipidemia due to adipocyte hypertrophy and hyperplasia [7]. In recent years, the mouse 3T3-L1 cells are often used as a model for studying the underlying mechanism of adipogenesis, which involves cell proliferation and differentiation [8,9]. The whole process of 3T3-L1 preadipocyte differentiation includes cell growth arrest, subsequent clonal expansion, and finally terminal differentiation into mature adipocytes [10]. So, it is beneficial for improving public health to clarify the potential molecular mechanisms of adipogenesis.

MicroRNAs are highly conserved endogenous short non-coding RNAs that work in the post-transcriptional regulation of gene expression [11,12], thereby repressing the translation of target genes and/or promoting their degradation [13]. It has been reported that miRNAs play crucial roles in many physiological and pathological processes, including energy homeostasis, tumorigenesis, development, glucose

metabolism, and lipid metabolism [14–19]. A recent study has indicated that miRNAs may play a functional role in regulating adipocyte differentiation and proliferation. For instance, miR-26b inhibits adipocyte differentiation and promotes proliferation in human preadipocytes [20]. The miR-375/miR-21/miR-143 cluster accelerates adipocyte differentiation in 3T3-L1 cells [21–23]. On the contrary, Let-7 and miR-27 impair adipogenesis [24,25]. In addition, miR-181a has been proven to regulate adipogenesis in the porcine model [26]. Therefore, miR-181a is one of the most representative miRNAs that regulates differentiation. However, the relationships between miR-181a-5p and the potential mechanisms of 3T3-L1 cell proliferation and differentiation remain unclear.

In our previous research, with the microarray strategy we found that miR-181a-5p is possibly a positive regulator during the chicken adipose tissue development [27]. However, to date, the role of miR-181a-5p in adipogenesis is still unknown.

The 3T3-L1 cell line has widely been used as an adipogenesis model and many signaling pathways have been proven to participate in 3T3-L1 cell differentiation, while the canonical Wnt signaling is a negative regulator during adipogenesis. Tumor necrosis factor-alpha inhibits adipogenesis via activating β-catenin/TCF4 (TCF7L2)-dependent signaling pathway [28]. Meanwhile, SMAD7 is a member of transforming growth factor-β (TGFβ)/Smad signaling pathway, which antagonizes the TGFβ-mediated response. Increased SMAD7 level blocks the differentiation of 3T3-F442A cells [29].

In this study, we identified whether miR-181a-5p can regulate the differentiation and proliferation of 3T3-L1 cells. Our results indicated that miR-181a-5p is an important positive regulator of adipogenesis by targeting *Tcf7l2* and *Smad7*. miR-181a-5p is involved in the differentiation and proliferation of 3T3-L1 cells, which may be related to the TGFβ/Smad signaling pathway. The decrease of *Tcf7l2* expression may promote 3T3-L1 cell differentiation by inhibiting Wnt/β-catenin signaling pathway. This study provides more insights into the mechanism of 3T3-L1 cell differentiation.

Materials and Methods

Cell culture and differentiation

3T3-L1 preadipocytes were cultured in DMEM (Gibco, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Gibco) in a 5% CO_2 atmosphere at 37°C. To induce the differentiation of 3T3-L1 preadipocytes, confluent preadipocytes were pre-treated for 2 days in differentiation medium: DMEM containing 10% FBS and MDI (8.61×10^{-7} M insulin, 1×10^{-6} M dexamethasone, and 5×10^{-4} M IBMX). Then, cells were washed with phosphate buffered saline (PBS) and were further cultured in DMEM containing 10% FBS and 8.61×10^{-7} M insulin (designated as Day 0).

Oil Red O staining and triglyceride content determination

According to the methods described previously [27], cells were fixed with 4% paraformaldehyde for 30 min. Then, the samples were washed twice with deionized water, and incubated with filtered Oil Red O (Sigma, St Louis, USA) working solution (60% Oil Red O stock solution and 40% deionized water) for 1 h. The stained cells were washed with isopropanol and the accumulated triglyceride was qualified by measuring the absorbance at 510 nm.

Transfection assays

The synthetic miR-181a-5p mimics (50 nM) and miRNA negative control (miR-NC) (RiboBio, Guangzhou, China) were transfected into 3T3-L1 cells by using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions.

RT-PCR and quantitative real-time PCR

Total RNA was extracted from 3T3-L1 preadipocytes using Trizol reagent (Takara, Otsu, Japan) according to the manufacturer's instructions. cDNA was synthesized by using ReverTra Ace PCR RT Master Mix with gDNA Remover (FSQ-301) (Toyobo, Osaka, Japan) and ReverTra Ace PCR RT Kit (Toyobo) for RT-PCR according to the manufacturer's protocol, respectively. In RT-PCR analysis, the mRNA levels of peroxisome proliferator-activated receptor gamma (*Pparγ*), CCAAT/enhancer-binding protein alpha (*C/ebpα*), fatty acid-binding protein 4 (*Fabp4*), *Adiponectin*, *Cdk4*, *Cdk6*, *CyclinD1*, *CyclinD2*, *CyclinD3*, *p21*, and *p53* were quantified using SYBR Green Supermix (Takara) with *β-actin* as a housekeeping gene for normalization. MiR-181a-5p level was measured by the stem-loop qPCR method [30], and U6 small nuclear RNA was used as an internal control. Data were calculated using the $2^{-\Delta\Delta CT}$ method. The sequences of all primers are listed in **Table 1**.

Table 1. Sequence of primers used in PCR

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
Pparγ	AAGAGCTGACCCAATGGTTG	ACCCTTGCATCCTTCACAAG
C/ebpα	CGCAAGAGCCGAGATAAAGC	CGGTCATTGTCACTGGTCAACT
Adipoq	ACGACCAGTATCAGGAAAAG	GGTAGAGAAGAAAGCCAGTAA
Fabp4	TAAAAACACCGAGATTTCCTTCA	CCTTTCATAACACATTCCACCA
β-actin	CATCCGTAAAGACCTCTATGCCAAC	ATGGAGCCACCGATCCACA
p21	CGAGAACGGTGGAACTTTGAC	CAGGGCTCAGGTAGACCTTG
p53	TTACCAGGGCAACTATGGCTTCC	CAACTGCACAGGGCACGTCTT
CyclinD1	ACCTCTGGCTCTGTGCCTTTCTAT	GTCCCACCTTCACCTCTTCCCT
CyclinD2	GAGTGGGAACTGGTAGTGTTGGGTA	CATGGCAAACTTGAAGTCGGTAGC
CyclinD3	AGATCAAGCCGCACAT	ATCCAGGTAGTTCATAGCC
Cdk4	CTACATACGCAACACCCG	TCAAAGATTTTCCCCAACT
Cdk6	CCTCTGGAGTGTCGGTTGC	CTGGGAGTCCAATGATGTCC
Tcf7l2 3′UTR	CGAGCTCTAGCAGACTGTCCAGAAAGCAT	GCGTCGACTAGGGAGACATTCAAAGTATTCAC

Western blot analysis

3T3-L1 preadipocytes were washed three times with ice-cold PBS, and then lysed with a RIPA buffer. The supernatants were collected by centrifuging at 12,000 *g* for 10 min at 4°C. Protein concentrations from cell lysates were measured by BCA kit (Beyotime, Haimen, China). Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes. The membranes were blocked for 1 h with 5% milk containing 0.1% Tween-20. Then, PVDF membranes were incubated with primary antibodies (all from Santa Cruz Biotech, Santa Cruz, USA) against PPARγ, C/EBPα, p-SMAD3, SMAD3, SMAD4, SMAD7, TLF7L2, c-MYC, P15, CDK4, CyclinD2, and Tubulin. Immune complexes were further incubated with specific horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotech), and the bands were visualized with BeyoECL Plus kit (Beyotime).

Cell viability assay

Cells were seeded into 96-well plates at 5×10^3 cells/well and then were transfected with miR-181a-5p mimics or NC. At 24 and 48 h after transfection, cell proliferation was determined using the CCK-8 kit (Beyotime) according to the manufacturer's instructions.

Luciferase vector construction and reporter assays

The mouse *Tcf7l2* was predicted to be the target of miR-181a-5p by using TargetScan and miRDB. The wild-type and mutant 3′UTR (RiboBio) of *Tcf7l2* mRNA were amplified from mouse cDNA with the primer containing the recognition sites of *Xho*I and *Sal*I listed in **Table 1**. The PCR product was inserted into the corresponding sites of pGL3-control (Promega, Madison, USA) to obtain the plasmid pGL3-Tcf7l2-UTR. For luciferase reporter assay, 3T3-L1 cells were co-transfected with miR-181a-5p mimics or miR-NC and the 3′UTR of pGL3-Wt-Tcf7l2 or pGL3-Mut-Tcf7l2 by using Lipofectamine 2000. After 48 h transfection, cells were collected and the luciferase activity was measured by using the Dual Luciferase Reporter Assay Kit (Promega).

Flow-cytometry analysis

Freshly prepared 3T3-L1 cells were digested with 0.25% trypsin (Gibco) and collected in PBS. Then, cells were fixed with 70% cold ethanol overnight at 4°C. Cells were washed with PBS twice and then stained with PI on ice. The cell suspensions were analyzed by flow cytometry immediately. The proportion of G0/G1, S, and G2/M phases was calculated from DNA histogram data [31].

Statistical analysis

All the experimental results were presented as the mean ± SEM. Data were analyzed using independent *t*-test. Differences were regarded as significant at a value of $P < 0.05$.

Results

The expression of miR-181a-5p during 3T3-L1 preadipocyte differentiation

To investigate the potential role of miR-181a-5p during the differentiation of 3T3-L1 preadipocytes into mature adipocytes, the expression level of miR-181a-5p mRNA was assessed by real-time PCR on Days 0, 2, 4, and 8 of differentiation. The result indicated that miR-181a-5p was gradually upregulated during 3T3-L1 preadipocyte differentiation (**Fig. 1**). So, we speculate that miR-181a-5p may have important function in 3T3-L1 preadipocyte differentiation.

miR-181a-5p overexpression induces the 3T3-L1 preadipocyte differentiation

To assess whether miR-181a-5p affects 3T3-L1 preadipocyte differentiation, we transfected miR-181a-5p mimics and miR-NC into 3T3-L1 cells. At 24 h after transfection, 3T3-L1 preadipocytes were induced to adipogenic differentiation with differentiation medium. Real-time qPCR assay showed that miR-181a-5p level was markedly increased in miR-181a-5p mimics group compared with the NC group at 24 h (**Fig. 2A**). MiR-181a-5p overexpression significantly promoted 3T3-L1 preadipocyte differentiation, which was confirmed by Oil Red O staining and triglyceride content measurement at 510 nm (**Fig. 2B,C**). On Day 8, compared with NC group, the expressions of *Pparγ*, *C/ebpα*, *Fabp4*, and *Adipoq* mRNA were markedly upregulated in miR-181a-5p mimics group during 3T3-L1 cell differentiation (**Fig. 2D**). Furthermore, the expression levels of PPARγ and C/EBPα protein were also increased (**Fig. 2E**). These data indicate that miR-181a-5p has a positive effect on 3T3-L1 preadipocyte differentiation.

miR-181a-5p overexpression inhibits proliferation of 3T3-L1 cells by inducing G1-phase cell-cycle arrest

Adipogenesis is involved in cell proliferation and differentiation. Meanwhile, the regulation of cell cycle plays an important role in cell proliferation [32]. In order to investigate the regulatory effect of miR-181a-5p on 3T3-L1 cell proliferation, miR-181a-5p mimics and miR-NC were successfully transfected into the 3T3-L1 cells (**Fig. 2A**). At 24 and 48 h after transfection, cell proliferation was assessed by CCK-8 assay and cell cycle was analyzed by flow cytometry. The results showed that miR-181a-5p overexpression significantly inhibited the proliferation of 3T3-L1 cells compared with NC (**Fig. 3A**). As shown in **Fig. 3B,C**, the miR-181a-5p group had a marked increase in G1-phase population compared with in the NC group. To explore the underlying mechanisms of miR-181a-5p in the cell-cycle arrest of 3T3-L1 preadipocytes, the mRNA and protein

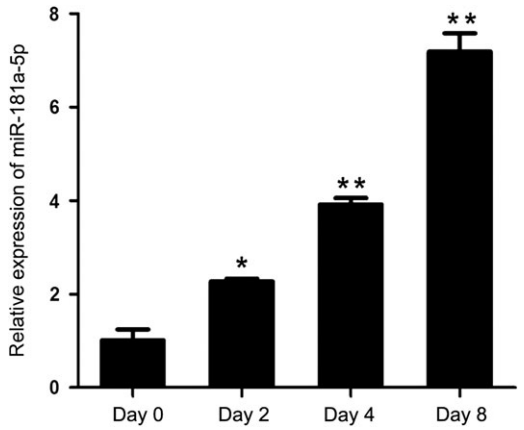

Figure 1. The expression of miR-181a-5p during 3T3-L1 preadipocyte differentiation 3T3-L1 preadipocytes were induced into differentiation as described in Materials and Methods. MiR-181a-5p was detected during 3T3-L1 cell differentiation at the designated time points by using real-time qPCR. $n = 3$. $^*P < 0.05$, $^{**}P < 0.01$.

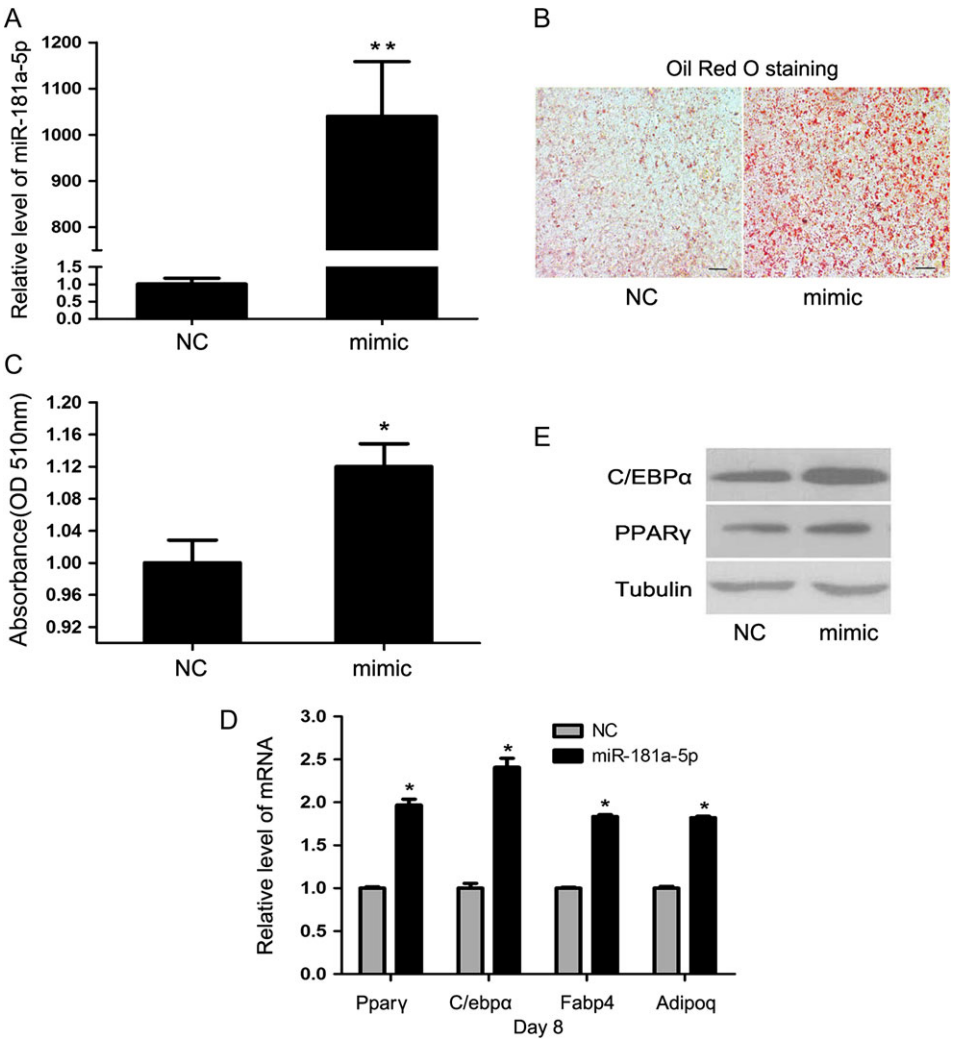

Figure 2. MiR-181a-5p promotes 3T3-L1 preadipocyte differentiation (A) MiR-181a-5p mimics or miRNA negative control (miR-NC) was transfected into 3T3-L1 cells, and at 24 h after transfection, miR-181a-5p transfection efficiency was confirmed by qPCR. (B) At 24 h post-transfection, the differentiation of 3T3-L1 preadipocytes was initiated. On Day 8 after differentiation, cells were fixed and stained with Oil Red O. (C) Triglyceride content was measured at 510 nm after extracting Oil Red O. (D) The relative mRNA expressions of adipocyte-specific molecular markers peroxisome proliferator-activated receptor gamma (*Pparγ*), CCAAT/enhancer-binding protein alpha (*C/ebpα*), fatty acid-binding protein 4 (*Fabp4*), and *Adipoq* were analyzed on Day 8 after the stimulation of differentiation. (E) The protein levels of PPARγ and C/EBPα were measured by western blotting. Scale bar, 100 μm. $n = 3$. $^*P < 0.05$, $^{**}P < 0.01$.

levels of G1 phase-related genes including *CyclinD1*, *CyclinD2*, *CyclinD3*, *CDK4*, *CDK6*, *p21*, and *p53* were detected. Results from qRT-PCR showed that supplementation of miR-181a-5p significantly suppressed the mRNA expressions of *CyclinD2*, *CyclinD3*, and *Cdk4*, but there was no significant difference in *CyclinD1*, *Cdk6*, *p21*, and *p53* (**Fig. 3D**). Western blot analysis showed that CyclinD2 and CDK4 expressions were downregulated and the expression of P15 was upregulated (**Fig. 3E**). These results indicate that miR-181a-5p inhibits cell proliferation as a result of G1-phase cell-cycle arrest.

Tcf7l2 and *Smad7* are the target genes of miR-181a-5p

In order to understand the underlying mechanism of miR-181a-5p in regulating preadipocyte differentiation, target prediction software TargetScan and miRDB were used to predict the potential target genes of miR-181a-5p. Prediction results revealed that *Tcf7l2* and *Smad7* may be direct target genes of miR-181a-5p. As shown in **Fig. 4A**, at 48 h after transfection, miR-181a-5p overexpression significantly reduced the expressions of TCF7L2 and SMAD7 protein in 3T3-L1 cells. According to a previous report, *Smad7* is a direct target of miR-181a, meanwhile miR-181a increases the epithelial–mesenchymal transition of ovarian cancer through the repression of Smad7 expression [33]. To further confirm that *Tcf7l2* is a target gene of miR-181a, we synthesized wild-type 3′UTR of mouse *Tcf7l2*-containing putative binding site for miR-181a-5p and the mutant 3′UTR with a 3-bp mutation in the seed region (**Fig. 4B**). The pGL3-Wt-Tcf7l2 and pGL3-Mut-Tcf7l2 luciferase reporter vectors including the binding site of miR-181a-5p were respectively co-transfected with miR-NC or miR-181a-5p mimics into 3T3-L1 cells. The results suggested that the miR-181a-5p overexpression significantly decreased the luciferase activity of the pGL3-Wt-Tcf7l2 when compared with pGL3-Mut-Tcf7l2, indicating that miR-181a-5p directly targeted *Tcf7l2* gene by binding to the 3′UTR (**Fig. 4C**). These data indicate that miR-181a-5p promotes 3T3-L1 cell adipogenesis by targeting *Smad7* [33] and *Tcf7l2*.

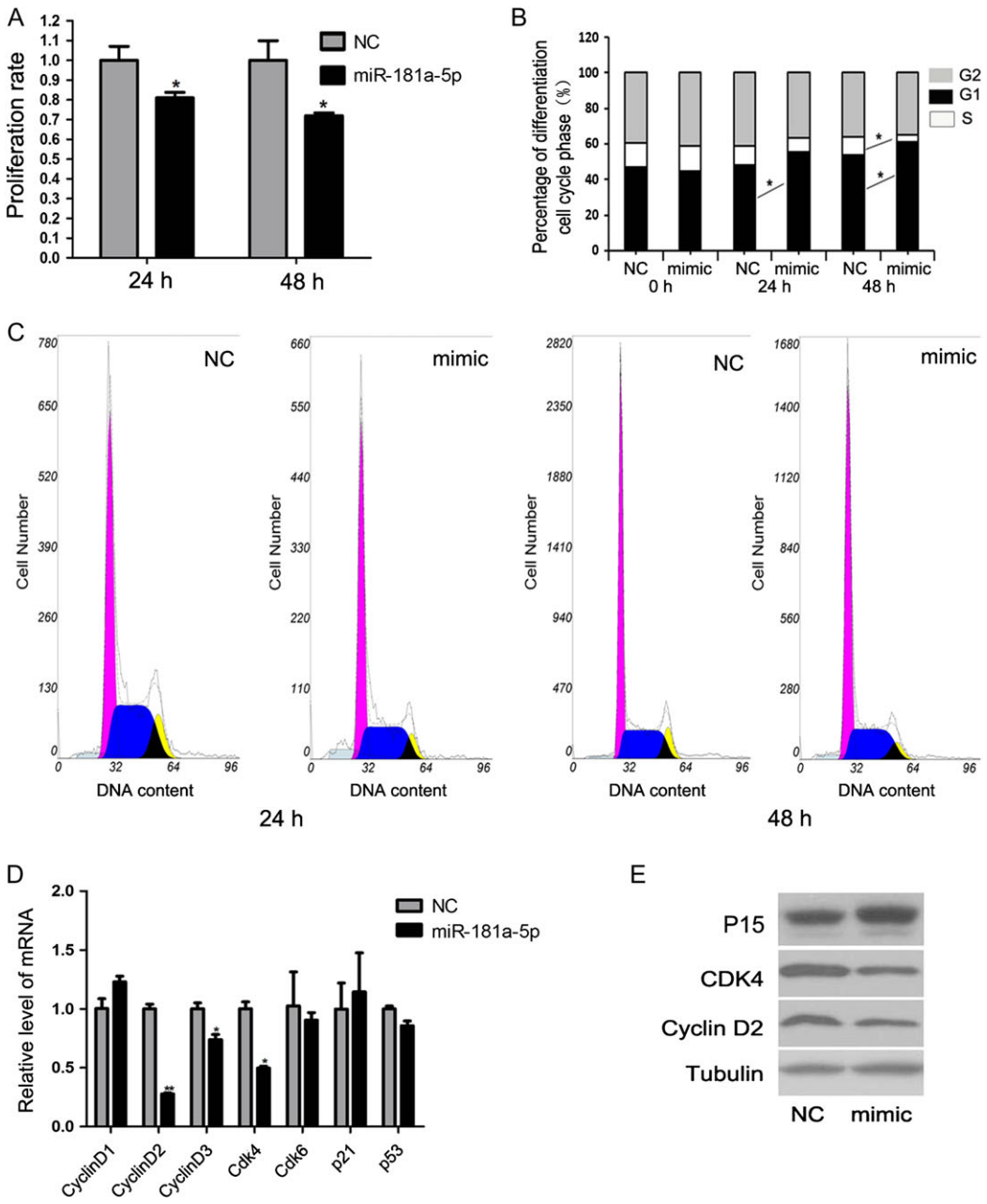

Figure 3. MiR-181a-5p inhibits proliferation of 3T3-L1 cells by regulating the cell cycle (A) 3T3-L1 cells were transfected with miR-181a-5p mimics or miRNA negative control (miR-NC). After transfection, cell proliferation was monitored at indicated time points. (B) After transfection, cells were harvested and determined the cell cycle by flow cytometry. (C) After transfection, cell cycle was analyzed. (D) At 48 h post-transfection, mRNA expression levels of cell G1 phase-related genes *CyclinD1, CyclinD2, CyclinD3, Cdk4, Cdk6, p21, and p53* were analyzed by qRT-PCR. (E) Western blot analysis showed that miR-181a-5p mimics downregulated the protein expression levels of CyclinD2 and CDK4 and upregulated the protein expression level of P15. Data are presented as the mean ± SD. $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$.

miR-181a-5p inhibits the TGFβ/Smad signaling pathway

In the TGFβ signaling pathway, SMAD7 inhibits the SMAD2/SMAD3/SMAD4 nuclear translocation by preventing their interaction. Meanwhile, TGFβ signaling pathway may be a negative regulator of adipogenesis [34]. To test whether miR-181a-5p regulates 3T3-L1 preadipocyte adipogenesis by influencing the downstream genes of Smad7 in the TGFβ signaling pathway, western blot analysis was performed to detect the expressions of p-SMAD3, SMAD3, SMAD4, and c-MYC at the protein level in TGFβ/Smad signaling pathway at 48 h after transfection with miR-181a-5p mimics and miR-NC. The expression of p-SMAD3 protein was upregulated in 3T3-L1 cells transfected with miR-181a-5p mimics compared with miR-NC groups, while the expression of SMAD3 was not affected (**Fig. 5**). On the contrary, miR-181a-5p overexpression significantly reduced the protein expression of SMAD4 and c-MYC (**Fig. 5**). These results suggest that miR-181a-5p overexpression regulates 3T3-L1 cell differentiation by influencing the protein expressions of p-SMAD3, SMAD4, and c-MYC in TGFβ/Smad signaling pathway probably by targeting *Smad7*.

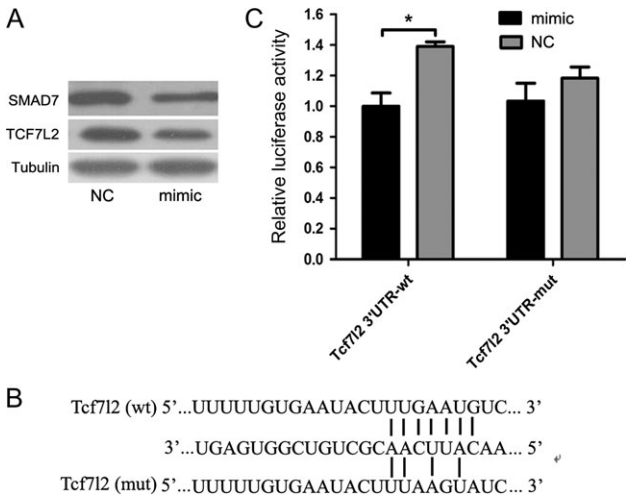

Figure 4. *Tcf7l2* and *Smad7* are direct target genes of miR-181a-5p in 3T3-L1 cells (A) Western blotting was performed to detect the protein level of TCF7L2 and SMAD7 at 48 h after transfection with miR-181a-5p mimics or miRNA negative control (miR-NC). (B) TargetScan analysis showed that *Tcf7l2* has the potential binding sites of miR-181a-5p in mouse. The mutated sites of 3′UTR of *Tcf7l2* were generated. (C) Wild-type or mutant *Tcf7l2* 3′UTR reporter constructs were transfected together with miR-181a-5p mimics or miR-NC into 3T3-L1 cells and then the relative luciferase activities were measured. Data are presented as the mean ± SD. $n = 3$. $^*P < 0.05$.

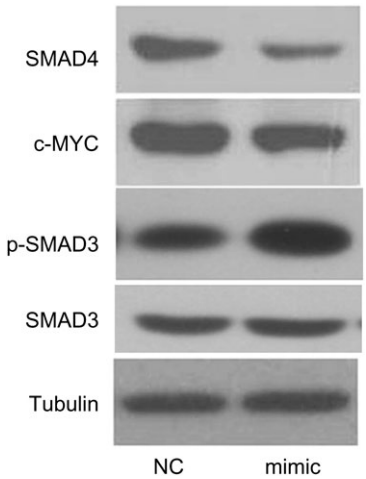

Figure 5. MiR-181a-5p regulates 3T3-L1 preadipocyte adipogenesis by influencing the downstream of target gene *Smad7* in the TGFβ signaling pathway The protein expressions of p-SMAD3, SMAD3, SMAD4, and c-MYC, which are the downstream genes of target gene *Smad7* in the TGFβ signaling pathway, were detected after transfection with miR-181a-5p mimics and miRNA negative control (miR-NC) at 48 h.

Discussion

In this study, we found that miR-181a-5p was gradually upregulated during the whole process of 3T3-L1 preadipocyte adipogenesis and that supplementation of miR-181a-5p significantly upregulated the mRNA expression of the adipogenic marker genes, such as *Pparγ*, *C/ebpα*, *Fabp4*, and *Adiponectin*, as well as the protein expressions of PPARγ and C/EBPα in 3T3-L1 preadipocytes. Our results indicate that miR-181a-5p may play a crucial role in 3T3-L1 preadipocyte adipogenesis. Recently, several reports have shown that miRNAs may directly or indirectly modulate various biological processes, such as adipocyte differentiation, cell proliferation, and tissue development [35,36]. For instance, the miR-17-92 cluster, miR-378a-3p, and miR-210 accelerate adipocyte differentiation [37–39]. In contrast, miR-138 and miR-155 inhibit adipogenic differentiation [40,41]. Meanwhile, it has been shown that miR-184 and miR-106 may be related to adipogenesis by regulating cell proliferation and cell-cycle arrest [42,43]. Most importantly, it has been reported that miR-181a can regulate adipogenesis in the porcine model [26]. All these findings imply that miR-181a-5p may serve as an important regulator during 3T3-L1 preadipocyte differentiation. In 3T3-L1 preadipocytes, due to the inverse correlation between differentiation and proliferation during the whole process of adipogenesis, we suppose that miR-181a-5p plays a negative role in 3T3-L1 cell proliferation. Our results showed that the transfection of miR-181a-5p mimics leads to cell-cycle arrest and significantly inhibits proliferation of 3T3-L1 cells. MiR-200a was significantly downregulated in hepatocellular carcinoma (HCC) cells, because miR-200a was found to inhibit the proliferation of HCC cells by induction of G1-phase arrest and CDK6 is a novel functional target of miR-200a [44]. Let-7c can inhibit HCC proliferation and induce cell-cycle arrest by directly targeting the cell-cycle regulator CDC25A [45]. TGFβ was found to increase the level of miR-424(322)/503 and reduce the CDC25A expression during cell-cycle arrest in mammary epithelial cells [46]. In human renal cell carcinoma, miR-99a inhibits the tumorigenicity by induction of G1-phase cell-cycle arrest [47]. These data suggest that some miRNAs may inhibit cell proliferation by inducing cell-cycle arrest.

Here, miR-181a-5p was identified as a novel miRNA that plays a role in 3T3-L1 preadipocyte differentiation and proliferation by targeting the *Smad7* gene in the TGFβ/Smad signaling pathway and *Tcf7l2* gene in the Wnt signaling pathway. MiRNAs have been demonstrated to regulate 3T3-L1 cell adipogenesis and related gene expression by targeting the TGFβ and Wnt signaling pathways [48].

Many previous studies indicated that miR-181a-5p played important roles in the breast cancer metastasis, the differentiation of osteoblasts, and the local immune balance by regulating the TGFβ signaling pathway [49–51]. Moreover, miR-181a regulates TGFβ signaling pathway by directly targeting *Smad7* gene to promote epithelial–mesenchymal transition [33], which plays a crucial role in the process of adipogenesis. This indicates that TGFβ signaling plays an important negative role in adipocyte differentiation [29]. SMAD7, a crucial member of the TGFβ signaling pathway, plays an important role in the regulation of adipocyte differentiation. TGFβ phosphorylates the SMAD2/SMAD3 by binding and activating the TGFβR1 and TGFβR2, which belong to transmembrane serine/threonine kinase receptors. The phosphorylated SMAD2/SMAD3 subsequently form complexes with SMAD4, and are finally translocated into the nucleus to regulate gene expression [52,53]. Our results demonstrated that downregulation of target gene *Smad7* induces the phosphorylation of SMAD3, but suppresses the expression of SMAD4. Thereby, the downregulation of complexes composed of phosphorylated SMAD2/SMAD3 and SMAD4 inhibits TGFβ signaling pathway. Suppressed TGFβ may promote adipogenesis and induce C/EBP transactivation function to increase their transcriptional activity on the promoters of key adipogenic genes [54]. It has been reported that TGFβ promotes cell proliferation of human embryonic palatal mesenchymal through activating both *Smad4* and *c-myc* genes during palatogenesis. SMAD4 induces *c-myc* gene activity through combination with Smad binding element in the c-myc

gene promoter [55]. In androgen receptor (AR)-negative prostate cancer, androgen induced 1 (PMEPA1), a prostate transmembrane protein, may promote AR-negative prostate cancer cell proliferation by suppressing SMAD3 phosphorylation and nuclear translocation, blocking the formation of SMAD3/SMAD4 complex, upregulating c-MYC, and downregulating P21 [56]. In addition, in the absence of TGFβ signaling, SMAD4 cooperates with lymphoid enhancer-binding factor 1/T cell-specific factor (LEF/TCF) to activate c-MYC expression in HepG2 cells [57]. So, SMAD4 promotes the expression of c-MYC by binding to its promoter in TGFβ signaling pathway. Our results also indicated that when miR-181a-5p inhibits the activity of SMAD7, downregulated SMAD4 suppresses the expression of c-MYC. Then, downregulated c-MYC has the capacity to inhibit cell proliferation after induction of p15INK4, which can inhibit CyclinD1–CDK4 interaction and then cause cell-cycle arrest [58–60]. Our results also showed that suppressed c-MYC may reduce the expression of CyclinD2/Cdk4 complexe to cause G1-phase cell-cycle arrest by promoting the expression of p15INK4 in TGF-β signaling pathway in 3T3-L1 cells. Previous studies also demonstrated that overexpression of c-myc gene could block the differentiation of mouse cells and inhibit the differentiation of 3T3-L1 cells by interrupting the entry of cells into a distinct predifferentiation stage in G0/G1 [61,62].

Tcf7l2 is a key regulator of Wnt signaling pathway whose function was demonstrated to suppress adipogenesis [63]. As a real target gene of miR-181a-5p, *Tcf7l2* was suppressed by supplementation of miR-181a-5p in 3T3-L1 cells. Downregulation of *Tcf7l2* can inactivate the transcription of Wnt-regulated genes by binding with β-catenin, which augments the expressions of C/EBPα and PPARγ to promote adipogenesis [64]. In the nucleus, TCF/β-catenin complexes can activate c-myc expression in the Wnt signaling pathway [65]. Therefore, downregulation of Tcf7l2 may inhibit the protein expression of c-myc in the Wnt signaling pathway, and then promote 3T3-L1 preadipocyte differentiation.

In summary, the present study suggests that miR-181a-5p regulates the differentiation and proliferation of 3T3-L1 by targeting the TGFβ and Wnt signaling pathways. Our study indicates that miR-181a-5p may be a potential target for ameliorating obesity, adipocyte hypertrophy, and hyperplasia.

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